

**EFFECTS OF CONCENTRATE ADDITION ON IN VITRO RUMEN
FLUID PH AND FORAGE FIBER DIGESTION**

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Abstract

It is common practice in the dairy industry to use a mixed concentrate and forage diet however, the ways in which feeds interact in the rumen to affect digestion are not well known. Further characterization could aid in the development of a more efficient diet. The objective of this study was to determine the early effects (0-6h) of concentrate type on rumen pH and forage digestion *in vitro*. An initial trial was conducted to compare the effect of buffer strength (full vs half) on pH change during *in vitro* digestion of orchard grass (*Dactylis glomerata* L.) with or without sucrose. It was determined that using a full strength buffer allowed for sufficient changes in pH for the purposes of this study. Two forages, orchard grass and corn (*Zea mays* L.) stover, were combined in a 50:50 ration with 5 concentrate treatments (no concentrate, corn meal, corn gluten meal, barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.)). The samples were incubated *in vitro* in a 1:4 buffer to rumen fluid mixture for 420 min. The pH was measured at intervals throughout the trial and samples were collected at the end for volatile fatty acid (VFA) analysis. Samples containing orchard grass and corn stover alone had a significantly lower pH ($P < 0.05$) than those samples containing concentrate beginning at T= 150 min and T=270 min respectively. For the corn stover samples, this correlates with a high mean lactic acid concentration (535 ppm) in comparison to the mean lactic acid concentration of the other four corn stover treatments (177 ppm). The final study measured the change in pH and the difference in neutral detergent fiber (NDF) digestibility of four concentrate treatments (no concentrate, corn meal, corn gluten meal, and barley). In addition, two sample distribution methods were tested; forage and concentrate were mixed together in the same filter bag or separated into individual bags. Barley had the largest inhibitory effect on fiber digestion and caused the largest decline in pH. The treatments with mixed feeds in the same bag showed larger differences in pH and digestibility. These results suggest that a diet containing a mixture of forages or, a mixture of corn and forage minimizes the decline in rumen pH in comparison with a single forage alone, mixed barley and forage, or mixed wheat and forage. They also suggest that the study of associative effects is impeded by separation of feeds through filter bags.

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Introduction

As global climate change effectuates a need to reduce green-house gas emissions and energy consumption, US agriculture systems will need to continue to reduce their environmental impact while still meeting demands for food products. The current dairy industry consists mainly of highly intensive systems, requiring large energy and monetary inputs. Decreasing the proportion of concentrate in dairy rations could reduce the monetary and environmental costs of these systems but a forage-based diet that can maintain current production levels is not known.

One approach to formulating new diets is through the use of digestion models, which predict animal responses to feeds. There have been many studies dedicated to improving the understanding of the complex processes involved in ruminant digestion using models based on data gathered from both *in vitro* and *in vivo* digestion trials. Modeling ruminant digestion with *in vitro* digestion systems can aid in the elucidation of specific aspects of digestion (such as changes in pH), leading to a better understanding of digestion mechanisms and thus better predictions of cow response to new diets. The interactions of different carbohydrate fractions provided by forages and concentrates is one particular area of interest, as their fermentation provides the cow's main source of energy.

The purpose of this study is to describe how different concentrates can alter the pH of the rumen and affect digestion of forages. A series of four experiments were conducted. The first *in vitro* trials were conducted to determine the appropriate buffer strength that allowed for sufficient fluctuations in pH using orchard grass as the forage and sucrose to simulate an energy source. The second trial repeated this two-buffer trial

using both orchard grass and corn stover to ensure the system was valid. The third trial used the system developed in the first two trials with five different concentrate treatments in order to compare the differences in pH changes. The fourth experiment used fermentation jars so that both changes in pH and digestibility through percent neutral detergent fiber (NDF) remaining could be measured.

Literature Review

Recent feeding trends have begun to reduce the proportion of concentrates in dairy-cattle diets after a period when higher milk yields were sought by increasing the amount of concentrate fed. This is in part due to the fact that increased production based on high-energy diets is not met without consequences. High concentrate diets increase the costs of production, require more energy to make, and increase the incidence of ruminal acidosis. Diets with larger proportions of forages could reduce these negative effects, but if production levels are to be maintained, research is required to find the appropriate forage-concentrate combination. In particular, the interactions of carbohydrate fractions, which can be modeled *in vitro*, require further study due to the fact that microbial fermentation of them provides the main source of energy.

Grass vs Grains

Ruminants have developed a digestive system that has a comparative advantage amongst the animal kingdom for digesting plant cell walls. Ruminant digestion is a result of a symbiotic relationship with the animal providing a stable environment and continuous supply of feed to a microbial population that is capable of converting the energy in otherwise indigestible celluloses into products that can be metabolized by the

ruminant (Van Soest, 1994). The domestication of ruminants by humans capitalized on this property of ruminant digestion by raising animals on grass-based diets to obtain animal products from otherwise unusable plant material. In contrast, the recent trend of supplementing ruminant diets with large proportions of concentrates depends on the ability of ruminants to utilize other energy sources such as simple sugars and starches, which are also energy sources for humans and other animals. Thus livestock and humans are competing for the same food energy sources, which can be seen as unethical in the face of persistent human malnutrition.

However, modern dairy and other ruminant production systems have been able to increase production/ animal in the past several decades in part due to increased proportions of cereal grains in diets (Fick and Clark, 1998; Van Soest, 1994). For example, the average milk production/cow for New York dairy farmers increased from 7372 kg in 1986 to 9202 kg in 1995 (Fick and Clark, 1998). Although the per cow milk production increased by roughly one fourth of the 1986 production levels, the average profitability/ cow dropped to nearly half of the original (Fick and Clark, 1998). Declines in profitability margins can be attributed to increases in purchased inputs, which largely consist of increases in purchased feedstuffs and inputs required to produce on-farm generated feeds.

In the case of imported grains, the large-scale production of corn in the American West supplies much of the demand for grain feedstuffs across the Americas and has resulted in transfers of soil nutrients across large areas of land. For example feed imports from Nebraska to Pennsylvania have resulted in nutrient excesses in Pennsylvania soils, which cause eutrophication of surface and groundwater (Fick and Clark, 1998). In

addition to environmental concerns associated with grain imports, the recent expansion of the biofuel market, in particular corn-based ethanol, has led to increases in the price of corn that will likely further decrease profitability in systems relying heavily on corn as a source of feed.

When considering on-farm concentrate production, the energy requirements for annual production and cultivation of cereal grains is far more than that of establishing a perennial grass sward that can then be managed in the future with very few energy inputs (Fick and Clark, 1998). Thus, replacing grasses with concentrates in ruminant diets has resulted in energy intensive systems that have added to the steady increase in American energy consumption, and in effect our contribution to global climate change.

Livestock production systems based on grazing have the potential to decrease pollution, energy needs, and input costs (Fick and Clark, 1998); however, as the demand for meat and dairy products will likely continue to rise, further study is needed to determine the most efficient use of concentrates and forages in ruminant nutrition so that the benefits of increased forage use can be seen without negative effects on production.

The need for accurate models

In order to make predictions of diets that allow for the most efficient use of grasses, accurate models of rumen digestion are needed. The first attempt to relate digestibility to feed composition was a regression equation published in 1938. This equation based digestibility on the proportion of crude fiber (CF) in the feed and its correlation to *in vivo* digestibility but was shown to have a high standard deviation (Minson, 1998). Better descriptors of nutritional quality were sought and it was found that the size of the standard deviation of the regression equation was dependent on the

chemical fraction used to make digestibility predictions (CF being a more accurate descriptor than crude protein (CP)), and the type of feed being described (Minson, 1998). The predicted digestibility of legumes was found to be more accurate than that of grasses and digestibility of mixed diets of legumes and grass was even less accurate (Minson, 1998). Recent attempts have been made to create mathematical models of rumen digestion using stoichiometric calculations based on the proportions of substrates in a feed and a few of the microbial groups (Nagorcka, 1999). These models' predictions have been proven to be inaccurate in part because they do not account for effects on pH or the wide variety of microbial compositions that effect VFA production rates. Thus they are not applicable for diet compositions that vary from those used to develop the model (Nagorcka, 1999, Minson, 1998).

One of the reasons accurate estimates of the nutritional quality of ruminant feeds are difficult to obtain is that diets generally consist of several components, which alter the digestion of each other. These interactions are known as associative effects. Orskov and Ryle (1990) described associative effects as "the reasons a mixed diet has a different nutritional value than the sum of the diets constituent feeds." So although assessments of the nutritional value of individual feeds exist, the nutritional value of a mixed diet is not an additive property. Due to the complexity of the rumen digestive system, which can include hundreds of different microorganisms in addition to the animal's digestive enzymes, characterizing these interactions is difficult. Lopez et al. (2000) describe a new mechanistic approach to rumen digestion modeling. They describe this type of mechanistic mathematical modeling as "looking at the structure of the system, dividing that structure into its key components and analyzing the behavior of the whole system in

terms of its individual components and their interactions with one another." Currently these models are only used in research because they show low levels of accuracy but their advantage is that new data can be added to the model at any time to improve their prediction accuracy (Lopez et al., 2000). Once these models obtain an acceptable level of accuracy it is believed they will be more generally applicable across a range of feed sources and environments than current models (Lopez et al., 2000).

***In vitro* digestion techniques**

Efforts to characterize the nutritional quality of feeds can be divided into *in vivo* and *in vitro* techniques. Advantages of using *in vitro* methods are that it is less time-consuming, less costly, and requires less feed than *in vivo* methods (Tamminga and Williams, 1998). Disadvantages of *in vitro* nutritional assessments relate to its accuracy in modeling the complex system of a live animal. Some areas where many *in vitro* techniques do not accurately simulate rumen digestion are the stratification of rumen contents *in vivo* in to liquid, solid and gas layers; the grinding of substrates for *in vitro* methods; the inability to simulate the removal of digestion end products *in vitro*; and the difference between the *in vivo* saliva buffering system and the *in vitro* pre-added buffer (Tamminga, and Williams, 1998).

In general there are two approaches to *in vitro* digestion systems. The first system incubates the feed substrates being studied with an inoculum of microbial population similar to those found in the rumen (Tamminga and William, 1998). This inoculum of microorganisms can come from collection of rumen fluid from fistulated animals, a culture of microorganisms obtained from either fresh or frozen feces, or effluent from a continuous fermentation culture. This method is better adapted to determining the energy

supply of the feed because of difficulties in distinguishing the protein that has been incorporated into the microbial protein mass and that which has been fermented for energy (Tamminga and Williams, 1998). The second approach incubates the feed with purified single or mixed preparations of enzymes. Aspects of ruminant digestion that can be studied using the inoculum method as opposed to the purified enzyme method are the effects of different diets on the microbial population and the changes to the rumen environment after feeding (Tamminga and Williams, 1998).

Rumen Digestion

Digestion in the rumen accounts for 60-70% of the total digestion in ruminants and is accomplished by the populations of microorganisms that benefit from a symbiotic relationship with their host animal. Fermentation is a means of energy production under anaerobic conditions and is the metabolic process used by microorganisms in animal digestive tracts. The rumen is thus analogous to a fermentation vat where microorganisms ferment the feed substrates ingested by the animal. The kinds and amounts of microorganisms present in the rumen vary greatly, but are generally divided into bacteria, protozoa and fungi. Bacteria are the most abundant microorganisms present in the rumen (on the order of 10^{10} - 10^{11} bacteria/ml) and represent about half of the microbial mass in the rumen (Orskov and Ryle, 1990, Van Soest, 1994). They are the most important contributors to rumen digestion (Van Soest, 1994). Because protozoa are much larger than bacteria, they may account for up to 40% of the microbial mass even though their relative numbers (10^5 - 10^6) and metabolic contribution are small (Orskov and Ryle, 1990, Van Soest, 1994). Fungi represent up to 8% of the rumen microbial mass though their metabolic contribution is poorly understood (Orskov and Ryle, 1990, Van

Soest, 1994). Carbohydrates are the main substrates used by rumen microorganisms to supply their energy needs, although soluble proteins can be hydrolyzed and the resulting amino acids can also be fermented for energy (Orskov and Ryle, 1990).

The protein content of a feed can be divided into two fractions in terms of their fate in the rumen. The first fraction is rumen degradable protein, which consists of any protein that is broken-down in the rumen before it is passed into the lower digestive tract. Once a protein has been hydrolysed into its constituent amino acids, they can either be incorporated into the microbial protein mass or fermented for energy into volatile fatty acids (VFA) and ammonia. The ammonia can then be used by the microbial population for *de novo* protein synthesis; a process that is favored by amylolytic bacteria (Beever and Mould, 2000). If excess ammonia is produced, it is absorbed across the rumen and excreted in the urine; precluding any advantages of feeding high protein diets. Microbial protein passes into the lower tract and is digested and absorbed by the host animal. In general, microbial protein provides the majority of the host's amino acid supply. The majority of forage protein is degraded in the rumen with an average from a sample of 52 forages of 720g/ kg CP being degraded in the rumen (Merchen and Bourquin, 1994). The other protein fraction is rumen undegradable protein, which consists of protein that passes through the rumen and is subsequently digested in the lower tract. Factors that affect the degradation of protein in the rumen include plant maturity, method of conservation, physical processing and whether the forage is a grass or a legume (Merchen and Bourquin, 1994).

Carbohydrates in the rumen can be divided into three main classes: water soluble carbohydrates, starches and structural carbohydrates. Water-soluble carbohydrates

(WSC) are simple sugars such as sucrose that are soluble in the rumen fluid. Water-soluble carbohydrates are found in the cell contents and do not significantly vary between forage species although the WSC content decreases with plant maturity (Beever and Mould, 2000). Most rumen microorganisms can utilize these small monosaccharides, disaccharides, and oligosaccharides (Orskov and Ryle, 1990). Starch, a form of energy storage for the plant, is composed of a varying ratio of amylose and amylopectin, neither of which is very soluble and both of which take longer to digest than simple sugars (Van Soest, 1994). Increasing proportions of amylopectin decreases the rate of starch digestion in the rumen, which is illustrated by the increase in rate of starch degradation from corn (higher proportion of amylopectin) to barley (lower proportion of amylopectin) (Beever and Mould, 2000). Grains such as corn and barley contain a large quantity of starch relative to grasses, and temperate grasses contain more starches than tropical grasses. Starches are fermented by amylolytic bacteria. The principal amylolytic bacteria are *Bacteriodes amylophilus*, *Selenomonas ruminatium*, and *Streptococcus bovis* (Theodorou and France, 1993). Structural carbohydrates consist of cellulose, hemicellulose and pectin; pectin being the most rapidly degraded and hemicellulose being the least rapidly degraded (Merchen and Bourquin, 1994). Pectin is not found in large quantities in grasses but can comprise more than 100g/kg of DM in legumes (Merchen and Bourquin, 1994). The main cellulolytic bacteria found in the rumen are *Bacteroides succinogenes*, *Ruminococcus albus*, *R. flavefaciens*, and *Eubacterium cellulosolvens* (Theodorou and France, 1993). Hemicellulose is degraded by some of the same species as those that degrade cellulose as well as *Butyrivibrio fibrisolvens* and *Bacteriodes ruminicola*

(Theodorou and France, 1993). Pectin is degraded primarily by *B. fibrosolvans* and *Lachnospira multiparus* (Theodorou and France, 1993).

In addition to microorganisms that digest the primary substrates provided by the feeds, there are those that ferment the metabolic products of this primary digestion. For example *Bacteroides succinogenes* ferments cellulose to produce succinate, acetate and formate. *Selenomonas ruminatum* then uses succinate in its metabolic reactions to produce propionate, acetate, and carbon dioxide (Van Soest, 1994). Another example of this type of microbial interaction is the production and utilization of hydrogen. The production of acetate and butyrate results in the production of hydrogen as well. Some of this hydrogen is used in the production of propionate but most of it is consumed in the process of methanogenesis in which carbon dioxide and hydrogen are converted to methane. Such interactions add to the complexity of the rumen digestion processes and partially explain the difficulties encountered when trying to model it.

Despite and in part due to these complexities, there are only five main products of microbial fermentation: acetate, propionate, butyrate, methane and carbon dioxide. Acetate, propionate, and butyrate represent the main VFA produced in the rumen and provide 50-80% of the host animal's energy (Merchen and Bourquin, 1994). Methane and carbon dioxide are waste gases that are eructated and account for most of the energy lost due to the process of fermentation. Thus the most efficient rumen feeding strategy will maximize VFA and microbial mass production while minimizing carbon dioxide and methane production.

VFA profiles

The proportions of the dominant VFA produced in the rumen vary with diet, microbial growth rates, level of feeding, and rumen pH (Lopez et al., 2000). High forage diets result in the production of higher amounts of acetate and butyrate while high starch diets result in the production of larger proportions of propionate even though acetate is still the dominant VFA (Beever and Mould, 2000). Propionate travels to the liver where it is converted to glucose. Acetate is mostly unchanged by the liver and supplies the main source of energy by either being oxidized to ATP or stored in long chain fatty acids. Acetate and butyrate are the significant contributors to long chain FA production for tissue deposition or secretion in milk. Conditions that inhibit methanogenesis increase the propionate: acetate ratio, while conditions that favor methanogenesis favor acetate production and increase energy losses to methane (Orskov and Ryle, 1990).

The effects of Rumen pH on Digestion

The pH of the rumen undergoes diurnal fluctuations and reflects the balance of acid production and absorption as well as the buffering function provided by bicarbonates in the saliva (Van Soest, 1994). After feeding, VFA production increases resulting in a depression in rumen pH. As the rate of VFA production decreases and absorption continues in the hours between feeding, the rumen pH will rise again. The rumen pH of cattle fed a predominantly forage diet is generally higher, in the range of 6.2-7, than those fed diets with larger proportions of concentrates such as US dairy cattle whose rumen pH ranges from 5.5-6.5 (Kolver, 2002).

The effect of rumen pH on digestion has been widely studied. Grant and Mertens (1992) found that the rate of fiber digestion is negatively affected below a pH of 6.2.

Yang and Beauchemin's (2002) more recent results agree with this finding, but state that activity of cellulolytic bacteria in particular is depressed when rumen pH falls below a pH of 6.2. Orskov and Ryle (1990) state that the reason for this depression in fiber digestion is a result of decreased multiplication of cellulolytic bacteria as well as inhibition of the process of cellulolysis itself. The inhibition of the process of cellulolysis is attributed to the sensitivity of cellulase to low pH (Stewart, 1977). Below a pH of 6 cellulolysis and cellulolytic bacteria multiplication are slowed and below a pH of 5.6 these processes are halted altogether (Orskov and Ryle, 1990). Many amylolytic bacteria, such as *S.bovi* have optimal pH ranges that are lower than those of their fiber-digesting counterparts (Orskov and Ryle, 1990). It has also been shown that depression total VFA production correlates with a low rumen pH (Yang and Beauchemin, 2002).

Under conditions of large available quantities of starch, *S.bovi* in particular can account for large drops in rumen pH. When growing slowly, *S. bovi* ferments starch into VFA. In contrast, when large quantities of starch are available *S. bovi* has the ability to grow much more rapidly and produces lactate instead of a VFA as its fermentation end product (Orskov and Ryle, 1990, Krauss and Oetzel, 2006). Thus as the pH of the rumen declines after feeding, the rumen environment shifts from favoring those bacteria that degrade fiber to those that degrade starches.

Outside of the effect that fermentation of starch has on the rumen pH, there is evidence to suggest that the type and composition of the carbohydrate component of the diet can affect the rate and extent of fiber digestion. Grant and Mertens (1992) found that the presence of starch decreases the rate of fiber digestion as well as increases the lag time. They believe that this effect is independent and multiplicative of the effect of a low

rumen pH on fiber digestion (Grant and Mertens, 1992). Mertens and Loften (1980) offered four hypotheses to explain the effect of starch on fiber digestibility:

1. Increase in digestion lag time
2. Decrease in rate of digestion
3. Decrease in potential extent of digestion
4. A combination of all or any of the first three

They found that addition of increasing quantities of starch resulted in a linear increase in digestion lag time (Mertens and Loften, 1980). Because the pH of the *in vitro* trials in this study was held constant, the increase in lag time is independent of the negative impacts of pH reduction. Tafaj et al. (2005) also found that reducing the concentrate level from 50% to 20% improved the digestibility of high-quality hay. Yang and Beauchemin (2001) found that the ratio of forage to concentrate did not affect the rumen pH; however the forage they used was alfalfa. As previously mentioned, legumes contain a large proportion of pectins which are more easily degraded than other structural carbohydrates and could account for the fact that no correlation was found between the level of concentrate and rumen pH or fiber digestion.

The experiments described in this thesis were conducted to further characterize the early (0-6h) interactions between forage, concentrates and rumen pH and how these factors relate to fiber digestibility. This early period of digestion was chosen because the period immediately after feeding is when the largest declines in rumen pH are seen. The effect of several types of concentrate on the pH of *in vitro* digestion trials was studied by taking pH readings at intervals throughout the 6 hr window to examine the rate of pH depression and how this is affected by forage-concentrate interactions.

Materials and Methods

Sample Preparation

Substrates were dried and ground through a 1-mm screen. Dry matter content was determined using 1 g samples dried for over 24 hr in a 105°C convection oven according to the procedure described by AOAC (Association of Official Agricultural Chemists) (1990).

In Vitro Procedure

Rumen fluid was collected at 9 am the morning of the digestion trials from a rumen-fistulated non-lactating cow fed medium quality Orchard grass hay and 1 kg of commercial 16% grain mixture. The ruminal fluid was strained initially through 4 layers of cheesecloth and again through 8 layers of cheesecloth. The ruminal fluid was purged with CO₂ before and during addition to samples to ensure an anaerobic environment. The full strength buffer with urea outlined by Marten and Barnes (1980) was used (Table 1). Buffer was added to the samples, followed by ruminal fluid in a 4:1 ratio, and CO₂ to displace the air inside the tubes or bottles.

Table 1. Buffer solution recipe. Solution A and Solution B are combined in a ratio of 50:1 the morning of the experiment.¹

| Solution A (g/liter distilled H₂O) | Solution B (g/ 100 ml distilled H₂O) |
|---|--|
| 10.0 KH ₂ PO ₄ | 15.0 Na ₂ CO ₃ |
| 0.5 Mg ₂ SO ₄ ·7 H ₂ O | 1.0 Na ₂ S·9 H ₂ O |
| 0.5 NaCl | |
| 0.1 CaCl ₂ ·2 H ₂ O | |
| 0.5 Urea | |

¹ Kansas State Buffer (Marten and Barnes, 1980).

Experiment 1

The objective of this preliminary trial was to determine the appropriate buffer strength to be used in future experiments. Orchard grass (*Dactylis glomerata L.*) was used as the substrate. 500 mg of each substrate was weighed into 100 ml plastic tubes. Buffer at a pH of 6.82 (40 ml) was added to half of the sample tubes and half-strength buffer (40 ml) at a pH of 6.93 was added to the other half. Sugar water (4 ml) containing 50 mM sucrose was added to half of the samples and 4 ml of water were added to the other half. Ruminal fluid (10 ml) was added to each tube. Samples were then purged with CO₂ before a stopper with a gas release valve was used to seal the tubes. Separate tubes were incubated for each time point. All of the tubes were swirled by hand.

The pH of the four different treatments (1/2 strength buffer + H₂O, 1/2 strength buffer + sugar, full strength buffer + H₂O, full strength buffer + sugar) was taken in duplicate at 30, 60, 90, 120, 150, 180, 300 and 420 minutes. All of the tubes (including those not being measured) were swirled by hand before each reading.

Experiment 2

The objective of this experiment was to conduct a repetition of the first trial to ensure the results were repeatable. The procedure of Experiment 1 was used with the addition of a corn stover (*Zea L.*) sample set using the same treatments. Readings were taken at 30, 60, 90, 120, 150, 180, 285 and 420 minutes.

Experiment 3

The objective of this experiment was to apply the methods established in the first trial to treatments that more closely simulated a cow's diet by using concentrate feeds for

the energy source instead of sucrose. The procedure from Experiment 1 was modified in the following ways. 500 mg of four different concentrate feeds (Corn Gluten Meal, Corn Meal, Barley (*Hordeum L.*), and Wheat (*Triticum L.*) were added in place of the sugar water to both orchard grass and corn stover samples. The pH readings were taken at 30, 90, 150, 210, 270 and 420 minutes in duplicate for each of the 10 treatments. Samples were frozen at 90, 210, and 420 minutes to be saved for future VFA analysis.

Fermentation analysis of grass haylage was performed by Dairy One (DHI Forage Testing Lab, Ithaca, NY). Forage samples, blended for 2 min in deionized water and filtered, were mixed 1:1 with 0.06 M oxalic acid. Samples were analyzed for acetic, propionic, butyric, and iso-butyric acids using gas chromatography (Anonymous, 1990). Lactic acid was determined using a YSI 2700 SELECT Biochemistry Analyzer equipped with an L-lactate membrane.

Experiment 4

The objective of this experiment was to measure pH changes during an established procedure used to determine NDF digestibility. The effect of eight concentrate treatments on pH and digestibility was studied by digesting samples in fermentation jars using the Daisy II 200/220 *in vitro* incubator (ANKOM Technology, Macedon NY). The buffer used was that described by Marten and Barnes (1980) with urea. Forage and concentrate samples were weighed into Ankom XT4 filter bags. Five forages were used (orchard grass, canary reed grass (*Phalaris arundinacea*), alfalfa (*Medicago sativa*), headed wheat and wheat straw) and were present in equal amounts in each of the eight treatments. Four concentrate treatments were used: corn gluten meal, corn meal, barley and no concentrate. The samples were distributed following the

procedure outlined in Figure 1. A total of 10 g of forage and 10 g of concentrate were added to each jar. Empty bags were sealed and added to the no-concentrate treatment to correct for losses of filter bag mass. 1.6 L of buffer and 400 ml of rumen fluid were added to each jar. The rumen fluid was collected according to the procedure described previously. The jars were incubated at 39°C and continuously rotated. Equal amounts (20 ml) of fluid were collected from each jar at 30 min and 6 hrs to measure the pH.

In vitro fiber digestibility was determined according to Cherney et al. (1983) using an ANKOM 200/220 fiber analyzer (ANKOM Technology, Macedon NY) except that the trial was stopped at 24 hr instead of 48 hr. The NDF digestibility was calculated using the following formula:

$$\text{NDF digestibility} = 1 - (\text{NDF remaining}/\text{NDF at } t=0)$$

The percent dry matter (DM) of the forages and concentrates used in Experiment 4 are listed in Table 2.

Table 2. Percent DM for substrates used

| | Corn Gluten Meal | Corn Meal | Barley | Orchard Grass | Alfalfa | Reed Canary Grass | Headed Wheat | Wheat Straw |
|------|------------------------|--------------|--------|------------------|---------|-------------------------|-----------------|----------------|
| % DM | 91.8 | 89.6 | 90.0 | 91.3 | 92.8 | 93.8 | 93.3 | 90.9 |

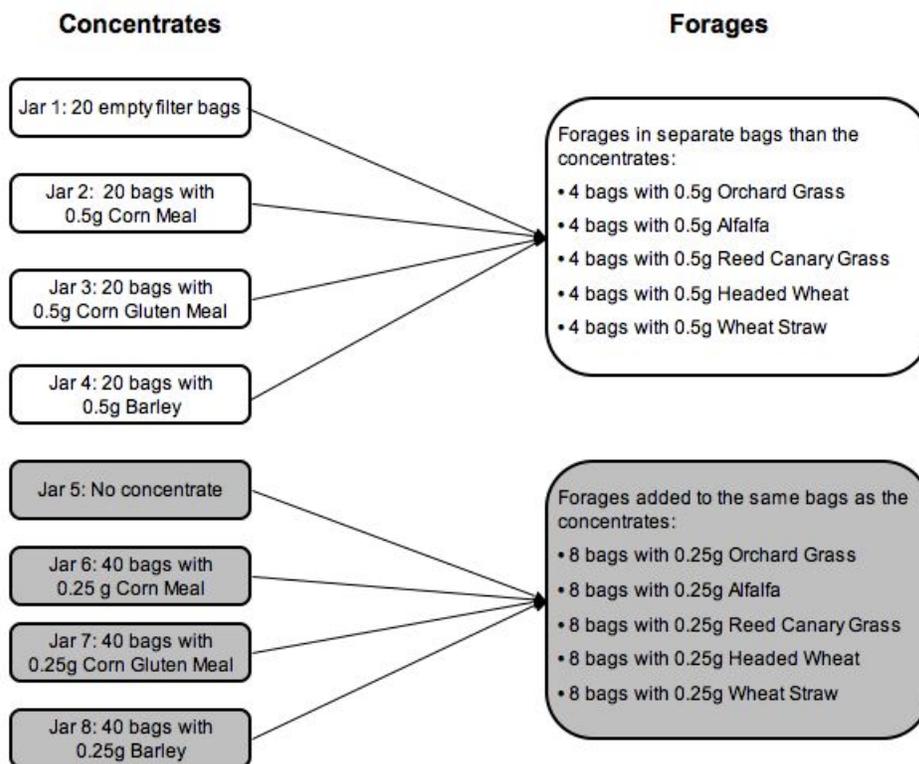


Figure 1: A schematic of treatment differences created through sample distribution for Experiment 4.

Statistical Analysis

Data was analyzed using repeated measures analysis models in the PROC MIXED procedure in SAS, version 7.0 software (SAS, 1998) according to Templeman and Douglass (1999). The covariance structure was assumed to be first order autoregressive, and degrees of freedom were calculated using the Satterthwaite method. Time is repeated with the subject being treatment.

Results and Discussion

Experiments 1 and 2

The addition of 50 mM sucrose solution to the orchard grass digestion tubes in Experiments 1 and 2 caused a significant ($P < 0.01$) drop of over 1 pH unit for both the

full and half strength buffer solutions (Fig 2 to 3). The difference in pH was significantly different ($P < 0.01$) dependent on sucrose addition beginning at $T=60$ min (Fig 2 to 3). In one case, the half-strength buffer fell below a pH of 5.5 (Fig 2), which is the low enough to cause ruminal acidosis and severely inhibit fiber digestion (Grant and Mertens, 1992, Krauss and Oetzel, 2006). The full-strength buffer treatments for the orchard grass samples in Experiment 1 and 2 dropped an average of 1.2 and 0.1 pH units from the initial to the final reading for the treatments with and without sucrose respectively (Fig 2 and 3). This indicates that the presence of a readily fermentable energy source such as sucrose causes an increase in acid production, which lowers the pH.

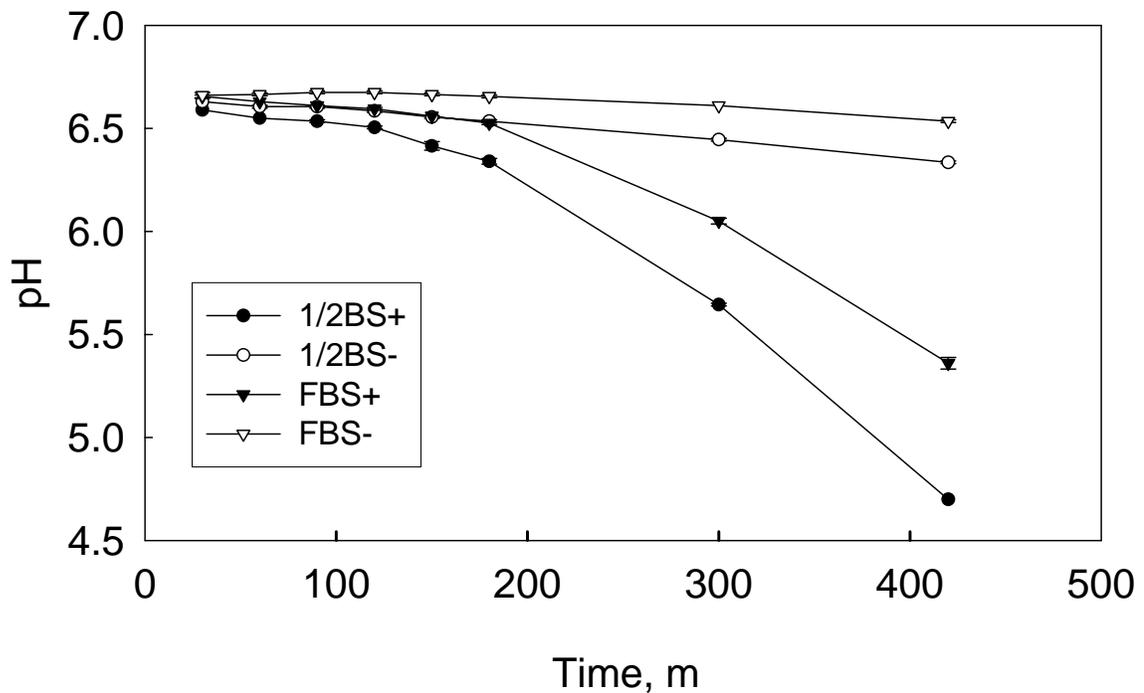


Figure 2. Comparison of the effects of sucrose addition to orchard grass on rumen fluid pH with varying strength buffer in Experiment 1. (1/2B=half-strength buffer, FB=full strength buffer, S+=with sucrose, S-=without sucrose).

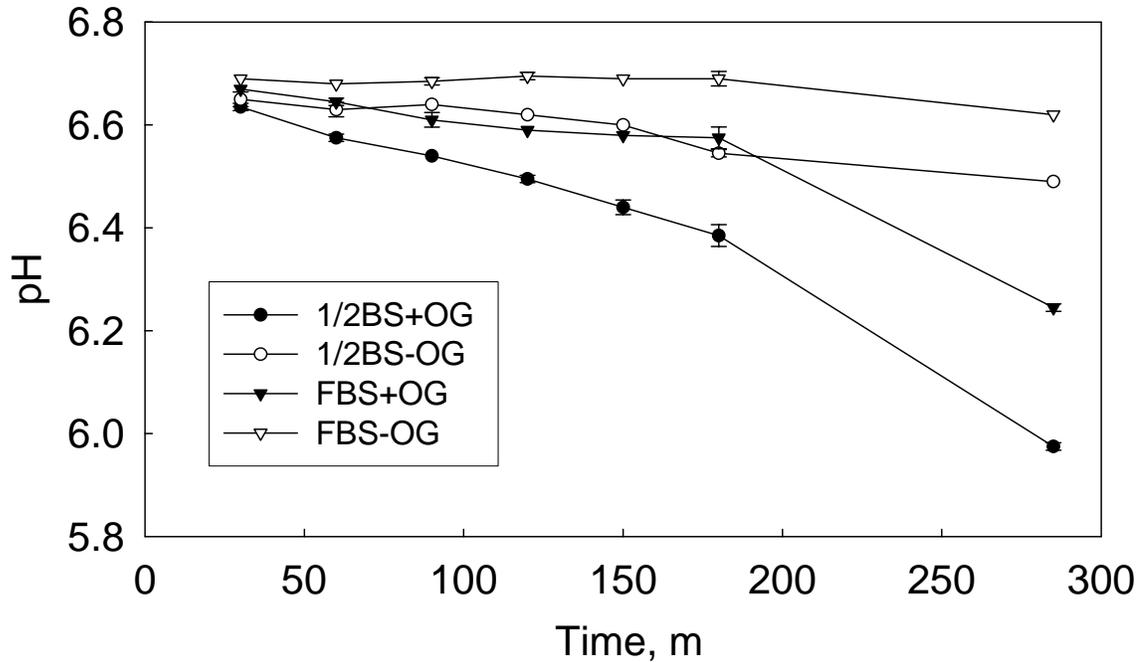


Figure 3. Comparison of the effects of sucrose addition to orchard grass on rumen fluid pH with varying strength buffer in Experiment 2. (1/2B=half-strength buffer, FB=full strength buffer, S+=with sucrose, S-=without sucrose, OG=orchard grass).

The addition of 50 mM sucrose to corn stover digestion tubes in Experiment 2 did not show as large of a decline as seen in the digestion of orchard grass (Fig 2 to 4). However the addition of sucrose still shows a significant difference ($P < 0.01$) in pH for both buffer treatments beginning at $T = 60$ min. The difference in the change in pH seen between the orchard grass and corn stover samples is likely attributable to differences in digestibility of the two forages. Also, the rumen fluid inoculum was obtained from a cow being fed a diet containing orchard grass which would mean the initial microbial population was likely more suited to digestion of orchard grass than corn stover. While, like the orchard grass samples, there was a larger decline in pH for the half-strength buffer treatments, the full-strength buffer treatments for the corn stover samples dropped

an average of 0.6 and 0.1 pH units from the initial to the final reading for the treatments with and without sucrose respectively (Fig 4).

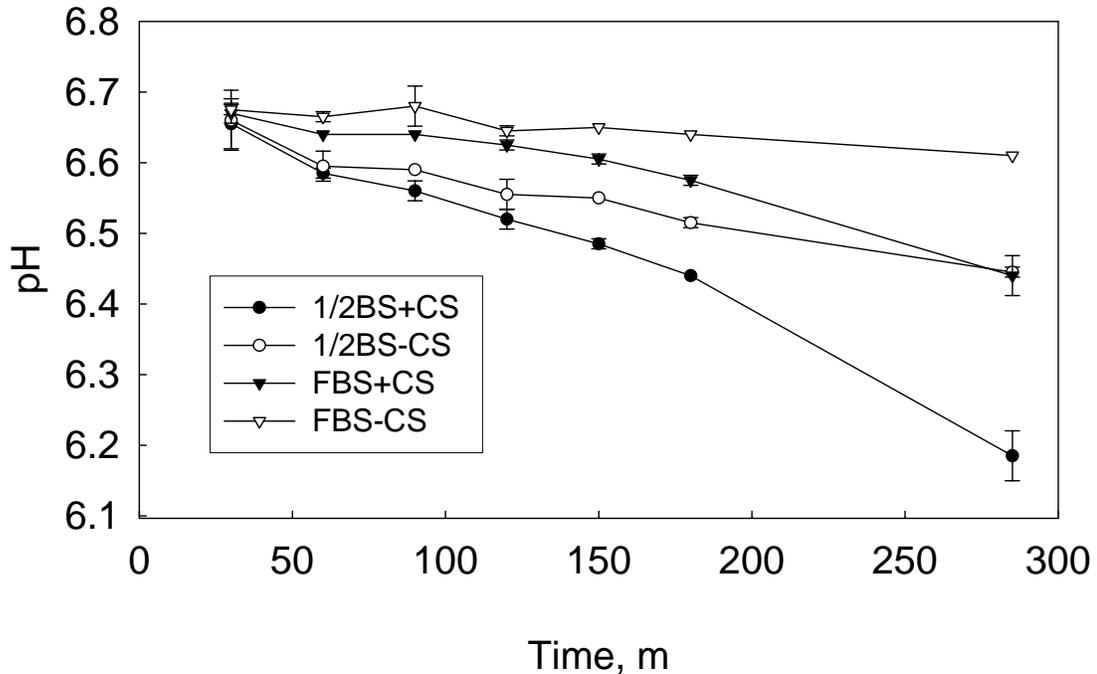


Figure 4. Comparison of the effect of sucrose addition to corn stover samples on rumen fluid pH with varying buffer strengths in Experiment 2. (1/2B=half-strength buffer, FB=full strength buffer, S+=with sucrose, S-=without sucrose, CS=corn stover).

Using sucrose as a substitute for a high-energy concentrate treatment clearly indicates that the presence of an easily digestible energy source decreases the rumen pH, which is in agreement with findings by Mertens and Loften (1980). Based upon the significant differences in pH seen using the full-strength buffer, it was deemed appropriate for the purposes of this study. Using the full strength buffer would allow for sufficient changes in pH between treatments and provide more buffering capacity to prevent the pH from dropping below normal *in vivo* rumen pH ranges.

Experiment 3

All treatments showed a decline in pH similar to those seen in the preliminary trials of Experiments 1 and 2; however, the drop in pH was not as dramatic as that seen with the addition of sucrose (Fig 2 to 6). As sucrose is water soluble and very readily fermented, it is not surprising that the addition of grain concentrates, which contain fractions of WSC, starches and structural carbohydrates, resulted in higher pHs; an indication of less acid production and thus less fermentation in that time period.

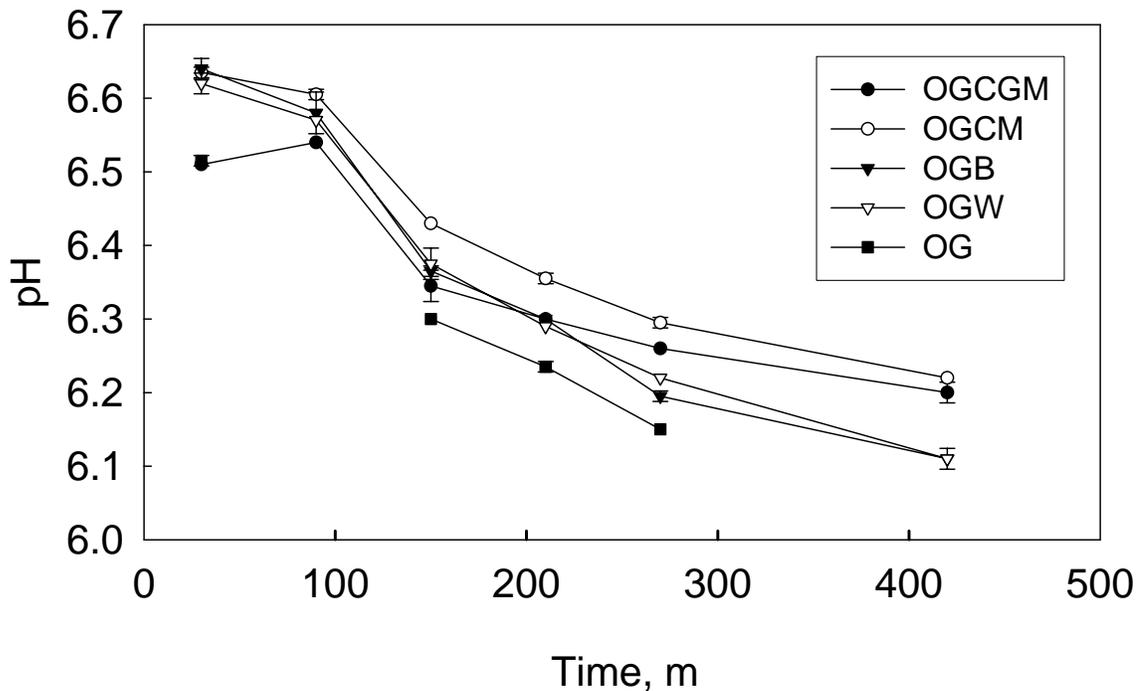


Figure 5. Comparison of the effects of the addition of five concentrate treatments to orchard grass samples on the pH of the rumen fluid. (OG= orchard grass, CGM= corn gluten meal, CM= corn meal, B= barley, W= wheat)

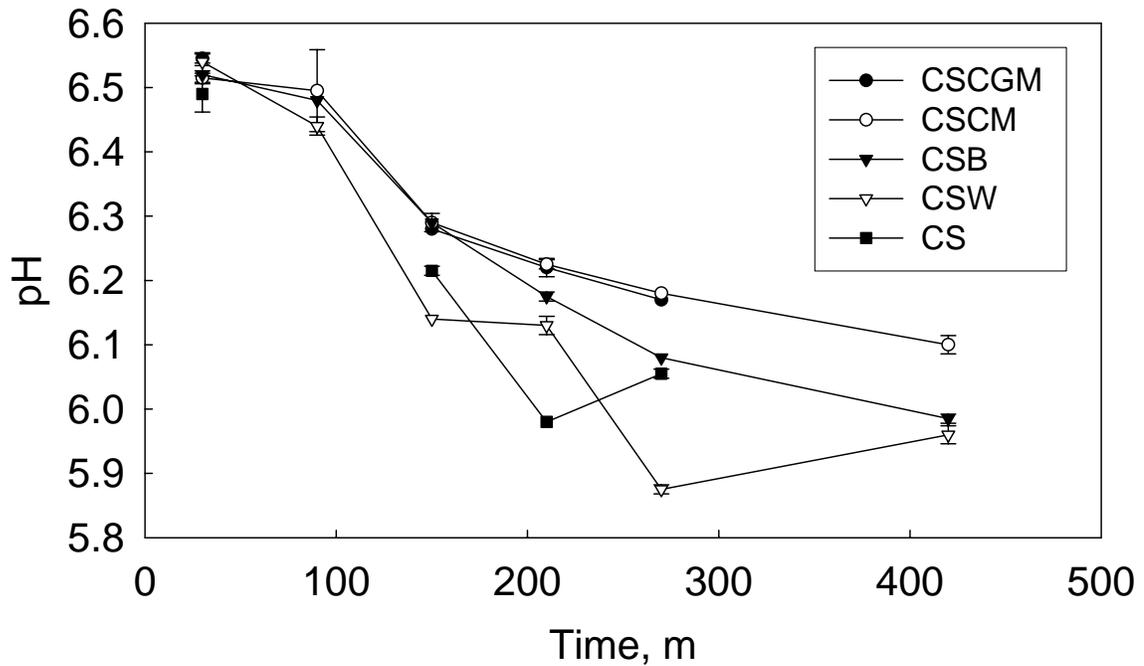


Figure 6. Comparison of the effects of the addition of five concentrate treatments to corn stover samples on the pH of the rumen fluid.

Contrary to what was expected, beginning at T=150 min the pH of the orchard grass treatment without concentrate was significantly lower ($P < 0.01$) than all of the concentrate treatments (Fig 5). In addition, the pH of the corn stover treatment without concentrate was significantly lower ($P < 0.01$) than all treatments except for wheat at T=150 min (Fig 6). Then at T=210, the corn stover sample pH was significantly lower ($P < 0.01$) than all treatments, similar to the pattern seen in the orchard grass sample set (Fig 5 and 6). However at T=270 min the corn stover pH increased, possibly due to microbial cell death as a result of a lack of nutrients. The low pHs seen in the corn stover sample set correspond with the highest concentration of lactic acid for both time points, although it is not statistically significant at T=30 min (Table 3). Because the pKa of lactic acid (pKa=3.08) is over 1 unit below the other common acids produced in the

rumen, its contribution to pH depression is exponentially larger than that of the other acids.

Table 3. Least squares mean of VFA concentrations for the corn stover sample set from Experiment 3 (ppm).

| Time | VFA | Corn stover | Corn stover + Barley | Corn stover + corn gluten meal | Corn stover + corn meal | Corn stover + wheat |
|---------|-----------------|--------------------|----------------------|--------------------------------|-------------------------|---------------------|
| 30 min | Lactic acid | 155 ^a | 134 ^a | 114 ^a | 122 ^a | 128 ^a |
| | Acetic acid | 920 ^a | 841 ^a | 849 ^a | 800 ^a | 876 ^a |
| | Propionic acid | 288 ^a | 276 ^a | 272 ^a | 259 ^a | 282 ^a |
| | IsoButyric acid | 35 ^a | 30 ^a | 35 ^a | 34 ^a | 35 ^a |
| | Butyric acid | 165 ^a | 160 ^a | 209 ^a | 153 ^a | 165 ^a |
| 270 min | Lactic acid | 535 ^c | 218 ^{a,b} | 146 ^{a,b} | 140 ^a | 225 ^b |
| | Acetic acid | 1132 ^a | 1173 ^a | 988 ^a | 1120 ^a | 1230 ^a |
| | Propionic acid | 422 ^{a,b} | 460 ^{a,b} | 384 ^a | 412 ^{a,b} | 493 ^b |
| | IsoButyric acid | 34 ^a | 36 ^a | 34 ^a | 35 ^a | 36 ^a |
| | Butyric acid | 220 ^a | 244 ^a | 209 ^a | 239 ^a | 254 ^a |

^{a,b,c} Means within a row with different superscripts differ ($P < 0.05$).

At T=270 min, the lactic acid concentration for the corn stover sample was more than double that of the corn stover with wheat sample (Table 3). Because the difference in concentrations of the other, weaker acids are much smaller, this provides support for the theory of cell death before this time point resulting in cell lyses and the release of neutralizing compounds that would raise the pH in spite of the large amounts of lactic acid. Although it was not expected that the pH of the forage samples without concentrate

would be the lowest or amongst the lowest, a possible explanation for this would be that the lack of competition from microorganisms digesting substrates from other feed sources allowed those digesting solely orchard grass or corn stover to thrive. In the case of corn stover, this resulted in the disproportionate production of lactic acid.

Table 4. Least squares means of VFA concentrations from the orchard grass sample set from Experiment 3 (ppm).

| Time | VFA | Treatment | | | | |
|---------|-----------------|-------------------|------------------------|----------------------------------|---------------------------|-----------------------|
| | | Orchard grass | Orchard grass + Barley | Orchard grass + corn gluten meal | Orchard grass + corn meal | Orchard grass + wheat |
| 30 min | Lactic acid | 176 ^b | 90 ^a | 96 ^{a,b} | 71 ^a | 87 ^a |
| | Acetic acid | 1078 ^a | 869 ^a | 794 ^a | 839 ^a | 848 ^a |
| | Propionic acid | 333 ^a | 276 ^a | 245 ^a | 263 ^a | 270 ^a |
| | IsoButyric acid | 37 ^a | 35 ^a | 33 ^a | 35 ^a | 35 ^a |
| | Butyric acid | 182 ^a | 157 ^a | 139 ^a | 153 ^a | 153 ^a |
| 270 min | Lactic acid | 138 ^a | 133 ^a | 63 ^a | 61 ^a | 108 ^a |
| | Acetic acid | 1293 ^a | 1161 ^a | 1109 ^a | 1024 ^a | 1213 ^a |
| | Propionic acid | 495 ^b | 433 ^{a,b} | 421 ^{a,b} | 349 ^a | 460 ^b |
| | IsoButyric acid | 37 ^a | 36 ^a | 35 ^a | 34 ^a | 37 ^a |
| | Butyric acid | 218 ^a | 219 ^a | 219 ^a | 186 ^a | 227 ^a |

^{a,b,c} Means within a row with different superscripts differ ($P < 0.05$).

Although it has been said that high forage diets produce an increased proportion of acetate and high concentrate diets produce an increased proportion of propionate, the results of this experiment show no significant difference ($P > 0.05$) in acetate, while the differences in propionate production do not follow this suggested pattern (Tables 3 and

4). Rather, the orchard grass samples without concentrate produced a significantly ($P < 0.05$) higher amount of propionic acid than the orchard grass with corn meal, and the corn stover samples without concentrate produced a significantly larger amount than the corn stover with corn gluten meal (Tables 3 and 4). The discrepancies in these results with the literature could be attributed to secondary fermentation of the initial fermentation products. While this does occur in the rumen, the absence of VFA removal through absorption during *in vitro* trials could influence the extent of these secondary fermentations. One way to analyze this further would be to take more frequent samples for VFA analysis over the course of the digestion trial.

In addition to the differences seen between concentrate and no-concentrate treatments, significant differences based on the type of concentrate were apparent. For the orchard grass sample set, barley and wheat treatments showed no significant differences ($P > 0.05$) over the entire trial (Fig 5). Although the pH of the corn stover sample with wheat was at times, significantly lower ($P < 0.05$) than that of the corn stover sample with barley, their pHs were consistently lower than the corn based concentrate treatments and their pHs converged at the last time point (Fig 6). These similarities in the pH of the wheat and barley treatments suggest comparable patterns of fermentation for these two concentrates. This is supported by the VFA profiles for the barley and wheat treatments. As seen in Tables 3 and 4, the VFAs and lactic acid concentrations were similar ($P > 0.05$) for each of the forages at both time points.

Just as wheat and barley treatments resulted in similar effects on pH, the corn meal and corn gluten meal treatment pHs were similar as well. For the corn stover sample set, corn meal and corn gluten meal treatments showed no significant differences

($P > 0.05$) over the entire trial (Fig 6). However, the pH of the corn gluten meal treatment was significantly lower ($P < 0.01$) than the corn meal treatment from T=30 min to T=150 min for the orchard grass sample set. As the trial proceeded, the difference in pH between these two treatments diminished and continuing from T=210 min there was not a significant difference between them. Like wheat and barley, the VFA and lactic acid concentrations were similar ($P > 0.05$) for both forages at both time points (Tables 3 and 4).

Experiment 4

Unlike the results from Experiment 3, the change in pH was the largest for the digestion jars treated with barley, not the treatments without a concentrate (Fig 7). A possible explanation for this discrepancy would be that this experiment used several types of forages. If the large declines in pH seen in the no-concentrate treatments in Experiment 3 are attributable to a lack of competition, then it is reasonable that the use of several different types of forages would provide a certain amount of competition, prevent the proliferation of one type or group of microorganisms, and thus prevent large drops in pH. Another explanation for the relatively small decline in pH for the no-concentrate treatments in Experiment 4 is that some of the forages used, such as the headed wheat and wheat straw were significantly less digestible than those used in Experiment 3 (Table 5).

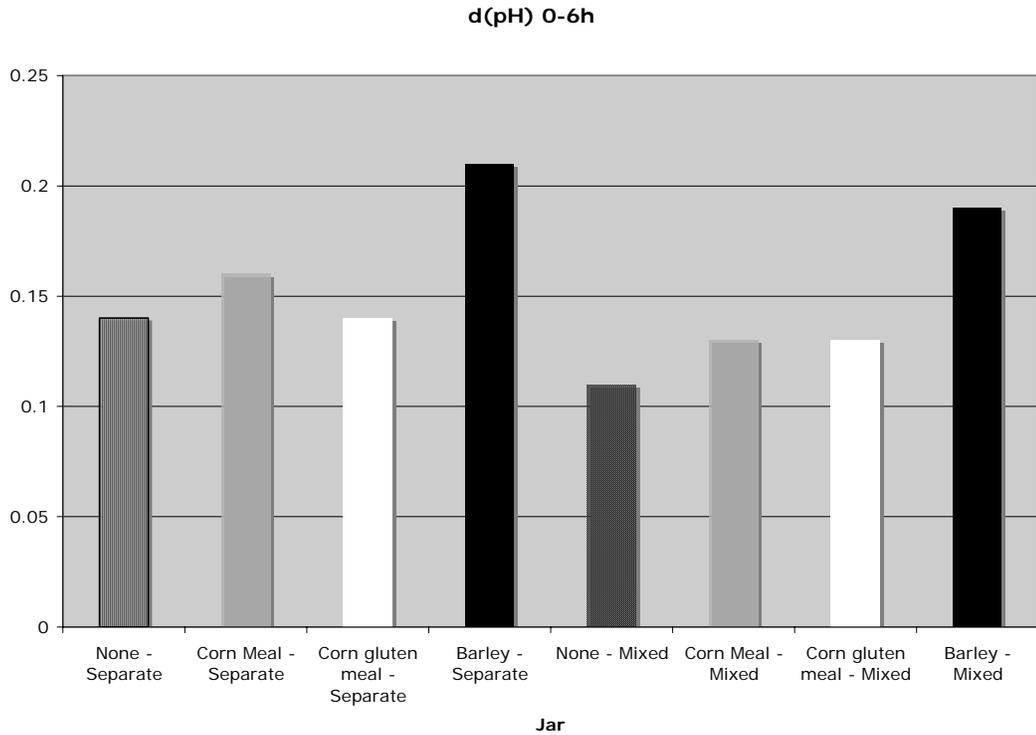


Figure 7. Change in pH from 0 to 6 hr in each of the 8 treatments from Experiment 4.

The changes in pH of the concentrate treatments however, are comparable to those seen in Experiment 3. The barley treatments decreased the pH more than either of the corn treatments (Fig 7). The difference in pH declines between barley (and wheat from Experiment 3) and the corn treatments suggest that barley (and wheat) has more readily fermentable carbohydrates than corn does, which is supported by Yang and Beauchemin's study (2001) that revealed similar results.

Table 5. Mean proportion of digestible NDF on a DM weight basis¹

| | 0.5 g Separate Sample | | | |
|-----------|--------------------------------|--------------------|--------------------|-------------------|
| | None | Corn Meal | CGM | Barley |
| OG | 0.78 ^b | 0.69 ^{ab} | 0.70 ^{ab} | 0.68 ^a |
| A | 0.71 ^a | 0.69 ^a | 0.70 ^a | 0.69 ^a |
| RC | 0.70 ^a | 0.73 ^a | 0.70 ^a | 0.70 ^a |
| WH | 0.60 ^b | 0.60 ^b | 0.57 ^{ab} | 0.56 ^a |
| WS | 0.53 ^b | 0.50 ^{ab} | 0.48 ^a | 0.48 ^a |
| Corn Meal | | 0.53 | | |
| CGM | | | 0.97 | |
| Barley | | | | 0.86 |
| | 0.25 g Separate Samples | | | |
| | None | Corn Meal | CGM | Barley |
| OG | 0.74 ^d | 0.56 ^b | 0.66 ^c | 0.50 ^a |
| A | 0.74 ^d | 0.60 ^b | 0.69 ^c | 0.52 ^a |
| RC | 0.78 ^d | 0.59 ^b | 0.69 ^c | 0.51 ^a |
| WH | 0.66 ^d | 0.49 ^b | 0.58 ^c | 0.41 ^a |
| WS | 0.56 ^c | 0.36 ^a | 0.46 ^b | 0.34 ^a |

¹NDF digestibility = 1- final NDF (g)/ initial NDF (g).

^{a,b,c} Means within a row with different superscripts differ (P<0.05)

(OG=orchard grass, A= alfalfa, RC= reed canary grass, WH= headed wheat, WS= wheat straw, CGM= corn gluten meal).

The presence of concentrate significantly decreased (P< 0.05) the forage NDF digestibility for all of the mixed samples and 3 out of 5 separated samples (Table 5). The decrease in digestibility is most likely not due to a decline in pH because the pH of the environment did not fall below 6.2, which has been suggested to be the point at which pH inhibits fiber digestion (Grant and Mertens, 1992, Yang and Beauchemin, 2002). Thus the presence of concentrates appears to have inhibited forage fiber digestion through some other mechanism. An increase in microbial competition leading to fiber digestion inhibition would explain the decrease in fiber digestion as well as the differences in digestibility seen between the separated and mixed samples (Table 5).

The differences in both pH depression and fiber digestion seen between the separately bagged forage and concentrate samples and the samples with forage and

concentrate in the same bag suggest the importance of microenvironments in the elucidation of associative effects. The separated sample treatments seemed to cause larger declines in pH than the mixed sample treatments (Fig 7). This larger pH depression could again be attributable to the decreased amount of competition in the microenvironments of the single-feed bags as compared to the mixed-feed bags. The digestibilities of the mixed samples were depressed in comparison with the separated samples (Table 5). The NDF digestibilities of the mixed feeds also showed more significant differences than those of the separated samples. Thus the microenvironment with a concentrate included in the same bag increased the inhibition of fiber digestion by a concentrate, which has been attributed specifically to starch described by Grant and Mertens (1992) and Mertens and Loften (1980).

Summary and Conclusion

It is evident from the results of these experiments that the presence of a concentrate in a ruminant diet influences the pH of the rumen fluid and to some extent inhibits the digestion of forage NDF. It is also clear that different kinds of concentrates have different effects on both of these factors. Barley and wheat cause larger declines in pH than corn meal or corn gluten meal probably due to larger amounts of easily fermented carbohydrates. In addition, barley has a larger inhibitory effect on forage fiber digestion than corn gluten meal, and corn gluten meal has a larger inhibitory effect on fiber digestion than corn meal. Through a comparison of the results from the different *in vitro* methods used, it is apparent that the method of digestion analysis influences the results. The procedure used in Experiments 1 through 3 may not be suitable for analysis of single feeds due to the fact that the forages showed larger pH declines and it is widely accepted that forage based diets maintain higher rumen pHs than diets with the inclusion of a concentrate. However, more trials should be conducted to confirm these results first. The results from Experiment 4 indicate that in order to study associative effects between feeds, the feeds must be in direct contact because separating them with filter bags at least diminishes these effects. Further studies should be conducted on the same day using the same substrates with both procedures to determine if there are differences in the change in pH that are attributable to the procedure alone. Conducting these experiments simultaneously and taking multiple digestibility measurements would also allow for a correlation of the rate of change in pH, VFA production and fiber digestion.

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